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UTILITY

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The compounds of Formula (I) are expected to inhibit the activity of Hepatitis C Virus NS3 protease. The NS3 protease inhibition is demonstrated using assays for NS3 protease activity, for example, using the assay described below for assaying inhibitors of NS3 protease. The compounds of Formula (I) are expected to show activity against NS3 protease in cells, as demonstrated by the cellular assay described below. Thus, the compounds of Formula (I) are potentially useful in the cure and prevention of HCV infections.

Expression and Purification of NS3 Protease

20 The plasmid cf1SODp600, containing the complete coding region of HCV NS3 protease, genotype 1a, was obtained from ATCC (database accession: DNA Seq. Acc. M62321, originally deposited by Chiron Corporation). PCR primers were designed that allow amplification of the DNA fragment encoding the NS3 protease catalytic domain (amino acids 1 to 192) as well as its two N-terminal fusions, a 5 amino acid leader sequence MGAQH (SEQ. ID. NO.:1) (serving as a expression tag) and a 15 amino acid His tag MRGSHHHHHMGAQH (SEQ. ID. NO.:2). The NS3 protease constructs were cloned in the bacterial expression vector under the control of the T7 promoter and transformed in *E. coli* BL 21 (DE3) cells.

30 Expression of the NS3 protease was obtained by addition of 1 mM IPTG and cells were growing for additional 3h at 25°C. The NS3 protease constructs have several fold difference in expression level, but exhibit the same level of solubility and enzyme specific activity. A typical 10 L fermentation yielded approximately 200 g of wet cell paste. The cell paste was stored at -80°C. The NS3 protease was purified based on published procedures (Steinkuhler C. et al. *Journal of Virology* 70, 6694-6700, 1996 and Steinkuhler C. et al. *Journal of Biological Chemistry* 271, 6367-6373, 1996.) with some modifications. Briefly, the cells were resuspended in lysis buffer (10 mL/g) containing PBS buffer

5 (20 mM sodium phosphate, pH 7.4, 140 mM NaCl), 50%
glycerol, 10 mM DTT, 2% CHAPS and 1mM PMSF. Cell lysis was
performed with use of microfluidizer. After homogenizing,
DNase was added to a final concentration 70 U/mL and cell
lysate was incubated at 4°C for 20 min. After
10 centrifugation at 18,000 rpm for 30 min at 4°C supernatant
was applied on SP Sepharose column (Pharmacia), previously
equilibrated at a flow rate 3 mL/min in buffer A (PBS
buffer, 10% glycerol, 3 mM DTT). The column was extensively
washed with buffer A and the protease was eluted by
15 applying 25 column volumes of a linear 0.14 - 1.0 M NaCl
gradient. NS3 containing fractions were pooled and
concentrated on an Amicon stirred ultrafiltration cell
using a YM-10 membrane. The enzyme was further purified on
26/60 Superdex 75 column (Pharmacia), equilibrated in
20 buffer A. The sample was loaded at a flow rate 1 mL/min,
the column was then washed with a buffer A at a flow rate 2
mL/min. Finally, the NS3 protease containing fractions were
applied on Mono S 10/10 column (Pharmacia) equilibrated in
50 mM Tris.HCl buffer, pH 7.5, 10% glycerol and 1 mM DTT
25 and operating at flow rate 2 mL/min. Enzyme was eluted by
applying 20 column volumes of a linear 0.1 - 0.5 M NaCl
gradient. Based on SDS-PAGE analysis as well as HPLC
analysis and active site titration, the purity of the HCV
NS3 1a protease was greater than 95%. The enzyme was stored
30 at -70°C and diluted just prior to use.

Enzyme Assays

Concentrations of protease were determined in the absence
of NS4a by using the peptide ester substrate Ac-
35 DED(Edans)EEAbuψ[COO]ASK(Dabcy1)-NH₂ (SEQ. ID. NO.:3)
(Taliani et al. *Anal. Biochem.* 240, 60-67, 1996.) and the
inhibitor, H-Asp-Glu-Val-Val-Pro-boroAla-OH (SEQ. ID.
NO.:5) and by using tight binding reaction conditions
(Bieth, *Methods Enzymol.* 248, 59-85, 1995). Best data was
40 obtained for an enzyme level of 50 nM. Alternately,
protease (63 µg/mL) was allowed to react with 3 µM NS4a,

- 5 0.10 mM Ac-Glu-Glu-Ala-Cys-pNA (SEQ. ID. NO.:4), and
varying level of H-Asp-Glu-Val-Val-Pro-boroAla-OH (0-6 μ M).
Concentrations of protease were determined from linear
plots of Activity vs. [inhibitor]. Molar concentrations of
proteases were determined from the x-intercept.
- 10 *ALL Cont.* K_m values were determined measuring the rate of
hydrolysis of the ester substrate over a range of
concentrations from 5.0 to 100 μ M in the presence of 3 μ M
KKNS4a (KKGSVVIVGRIVLSGKPAIIPKK) (SEQ. ID. NO.:6). Assay
were run at 25°C, by incubating ~1 nM enzyme with NS4a for 5
15 min in 148 μ l of buffer (50 mM Tris buffer, pH 7.0, 50%
glycerol, 2% Chaps, and 5.0 mM DTT. Substrate (2.0 μ l) in
buffer was added and the reaction was allowed to proceed
for 15 min. Reactions were quenched by adding 3.0 μ L of 10%
TFA, and the levels of hydrolysis were determined by HPLC.
- 20 Aliquots (50 μ L) were injected on the HPLC and linear
gradients from 90% water, 10% acetonitrile and 0.10 % TFA
to 10% water, 90% acetonitrile and 0.10% TFA were run at a
flow rate of 1.0 mL/min over a period of 30 min. HPLCs were
run on a HP1090 using a Rainin 4.6 x 250 mm C18 column (cat
25 # 83-201-C) fluorescent detection using 350 and 500 nm as
excitation and emission wavelengths, respectively. Levels
of hydrolysis were determined by measuring the area of the
fluorescent peak at 5.3 min. 100% hydrolysis of a 5.0 μ M
sample gave an area of 7.95 ± 0.38 fluorescence units.).
- 30 Kinetic constants were determined from the iterative fit of
the Michaelis equation to the data. Results are consistent
with data from Liveweaver Burk fits and data collected for
the 12.8 min peak measured at 520 nm.
- Enzyme activity was also measured by measuring the
35 increase in fluorescence with time by exciting at 355 nm
and measuring emission at 495 nm using a Perkin Elmer LS 50
spectrometer. A substrate level of 5.0 μ M was used for all
fluorogenic assays run on the spectrometer.

40 Inhibitor Evaluation In vitro

5 Inhibitor effectiveness was determined by measuring enzyme activity both in the presence and absence of inhibitor. Velocities were fit to the equation for competitive inhibition for individual reactions of inhibitors with the enzyme using

10
$$v_i / v_o = [K_m (1 + I/K_i) + S] / [K_m + S].$$

all cont.
15 The ratio v_i / v_o is equal to the ratio of the Michaelis equations for velocities measured in the presence (v_i) and absence (v_o) of inhibitor. Values of v_i / v_o were measured over a range of inhibitor concentrations with the aid of an Excel™ Spreadsheet. Reported K_i values are the average of 3-5 separate determinations. Under the conditions of this assay, the IC_{50} and K_i s are comparable measures of inhibitor effectiveness.

20 Using the methodology described above, compounds of the present invention were found to exhibit a K_i of $\leq 60 \mu M$, thereby confirming the utility of the compounds of the present invention as effective NS3 protease inhibitors. Preferred compounds of the present invention have K_i 's of $\leq 1 \mu M$. More preferred compounds of the present invention
25 have K_i 's of ≤ 100 nM. Most preferred compounds of the present invention have K_i 's of ≤ 10 nM.

Inhibitor Evaluation in Cell Assay.

30 The following method was devised to assess inhibitory action of test compounds on the HCV NS3 protease in cultured cells. Because it is not possible to efficiently infect cells with hepatitis C virus, an assay was developed based on co-expression in transfected cell lines of two plasmids, one is able to direct synthesis of the NS3
35 protease and the other to provide a polypeptide analogous to a part of the HCV non-structural protein containing a single known peptide sequence highly susceptible to cleavage by the protease. When installed in cultured cells by one of a variety of standard methods, the substrate
40 plasmid produces a stable polypeptide of approximately 50KD, but when the plasmid coding for the viral protease is

5 co-expressed, the enzymatic action of the protease
hydrolyzes the substrate at a unique sequence between a
cysteine and a serine pair, yielding products which can be
detected by antibody-based technology, eg, a western blot.
Quantitation of the amounts of precursor and products can
10 be done by scanning film auto-radiograms of the blots or
direct luminescence-based emissions from the blots in a
commercial scanning device. The general organization of the
two plasmids is provided in Scheme 6. The coding sequences
for the NS3 protease and the substrate were taken from
15 genotype 1a of HCV, but other genotypes, eg 2a, may be
substituted with similar results.

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The DNA plasmids are introduced into cultured cells
using electroporation, liposomes or other means. Synthesis
of the protease and the substrate begin shortly after
20 introduction and may be detected within a few hours by
immunological means. Therefore, test compounds are added at
desired concentrations to the cells within a few minutes
after introducing the plasmids. The cells are then placed
in a standard CO₂ incubator at 37°C, in tissue culture
25 medium eg Dulbecco-modified MEM containing 10% bovine
serum. After 6-48 hours, the cells are collected by
physically scraping them from plastic dishes in which they
have been growing, centrifuging them and then lysing about
10⁶ of the concentrated cells in a minimal volume of
30 buffered detergent, eg 20 µl of 1% sodium dodecyl sulfate
in 0.10 M Tris-HCl, pH 6.5, containing 1% mercaptaethanol
and 7% glycerol. The samples are then loaded onto a
standard SDS polyacrylamide gel, the polypeptides separated
by electrophoresis, and the gel contents then
35 electroblotted onto nitrocellulose or other suitable paper
support, and the substrate and products detected by
decoration with specific antibodies.

Although this invention has been described with
respect to specific embodiments, the details of these
40 embodiments are not to be construed as limitations. Various
equivalents, changes and modifications may be made without

5 departing from the spirit and scope of this invention, and
it is understood that such equivalent embodiments are part
of this invention.

10 Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg pinanediol
ester•trifluoroacetate (SEQ. ID. NO.:7).

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Good*
15 Preparation of Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH (SEQ.
ID. NO.:8). Boc-Val-Pro-OBzl was prepared by dissolving H-
Pro-OBzl (20 g, 83 mmol) in 50 mL of chloroform and adding
Boc-Val-OH (18.0 g, 83 mmol), HOBT (23.0g, 165 mmol), NMM (9.0 mL, 83 mmol) and DCC (17.0 g, 83 mmol). The reaction
mixture was stirred overnight at room temperature. The
mixture was filtered and solvent was evaporated. Ethyl
acetate was added and insoluble material was removed by
20 filtration. The filtrate was washed with 0.2N HCl, 5%
NaHCO₃, and saturated aqueous NaCl. It was dried over
Na₂SO₄, filtered and evaporate to give a white solid (30 g,
75 mmol, 90%). ESI/MS calculated for C₂₂H₃₂N₂O₅ +H: 405.2.
Found 405.6.

25 Boc-Val-Val-Pro-OBzl was prepared by dissolving Boc-Val-
Pro-OBzl (14.0 g, 35.0 mmol) in 4N HCl in dioxane (20 mL)
and allowing the reaction to stir for 2h under an inert
atmosphere at room temperature. The reaction mixture was
30 concentrated by evaporation *in vacuo* and ether was added to
yield a precipitate. It was collected by filtration under
nitrogen. After drying *in vacuo* with P₂O₅, H-Val-Pro-OBzl
was obtained as a white solid (22.6 g, 30.3 mmol, 89%).
(ESI/MS calculated for C₁₇H₂₄N₂O₃ +H: 305.2. Found:
35 305.2.) H-Val-Pro-OBzl (9.2 g, 27 mmol) was dissolved in
50 mL of CH₂Cl₂ and Boc-Val-OH (7.3 g, 27 mmol), HOBT (7.3
g, 54 mmol), NMM (3.0 mL, 27 mmol) and DCC (5.6 g, 27 mmol)
were added. The reaction mixture stirred overnight at room
temperature. The mixture was filtered and the filtrate was
40 evaporated. The residue was dissolved in ethyl acetate and
the solution was re-filtered. The filtrate was washed with

5 0.2N HCl, 5% NaHCO₃, and saturated aqueous NaCl. It was dried over Na₂SO₄, filtered and evaporated to give a yellow oil (10.6 g, 21.1 mmol, 78%). ESI/MS calculated for C₂₇H₄₁N₃O₆ + Na: 526.3 Found: 526.4.

10 Z-Glu(O^tBu)-Val-Val-Pro-OBzl (SEQ. ID. NO.:9) was also prepared by DCC coupling. H-Val-Val-Pro-OBzl•hydrochloride was obtained in a 100% yield by treating the corresponding Boc compound with anhydrous HCl using the procedure described for H-Val-Pro-OBzl (ESI/MS calculated for C₂₂H₃₃N₃O₄ + H: 404.2. Found 404.3.). The amine hydrochloride (7.40 g, 16.8 mmol) was dissolved in 185 mL DMF and 25 mL THF. Z-Glu(O^tBu)-OH (5.60 g, 16.8 mmol), HOBt (4.60 g, 33.6 mmol), NMM (1.85 mL, 16.8 mmol) and DCC (3.5 g, 16.8 mmol) were added. The reaction was run and
15 the product was isolated by the procedure described for Boc-Val-Val-Pro-OBzl. The tetrapeptide was obtained as a white foam (12.0 g, 16.1 mmol, 96%). ESI/MS calculated for C₃₉H₅₄N₄O₉ + Na: 745.4. Found: 745.4.

25 H-Glu(O^tBu)-Val-Val-Pro-OH (SEQ. ID. NO.:10) was prepared by dissolving Z-Glu(O^tBu)-Val-Val-Pro-OBzl (2.90 g, 3.89 mmol) in 100 mL methanol containing 1% acetic acid. Pearlman's catalyst, Pd(OH)₂, (100mg) was added and the flask was placed on the Parr hydrogenation apparatus with
30 an initial H₂ pressure of 34 psi. After three hours, the catalyst was removed by filtration through a celite pad and the filtrate was evaporated in vacuo to yield a yellow oil (1.30 g, 2.61 mmol, 67%). ESI/MS calculated for C₂₄H₄₂N₄O₇ +H: 499.3 Found: 499.4.

35 Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH was prepared by active ester coupling. Boc-Asp(O^tBu)-N-hydroxysuccinimide ester was prepared by coupling Boc-Asp(O^tBu)-OH (3.00 g, 10.4 mmol) to N-hydroxysuccinimide (1.19 g, 10.4 mmol) in
40 50 mL of ethylene glycol dimethyl ether. The reaction flask was placed in an ice bath at 0°C and DCC was added.

- 5 The reaction mixture was slowly allowed to warm to room temperature and to stir overnight. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and re-filtered. The filtrate was evaporated give a white solid.
- 10 Recrystallized from ethyl acetate: hexane gave the activated ester (3.38 g, 8.80 mmol, 84%). (ESI/MS calculated for $C_{17}H_{26}N_2O_8 + H$: 387.2. Found: 387.4.) H-Glu(O^tBu)-Val-Val-Pro-OH (5.40 g, 10.8 mmol) was dissolved in 100 mL of water. Sodium bicarbonate (0.92 g, 11.0 mmol) was added followed by triethylamine (2.30 mL, 16.5 mmol). The N-hydroxysuccinimide ester (3.84 g, 10.0 mmol) was dissolved in 100 mL dioxane and was added to the H-Glu(O^tBu)-Val-Val-Pro-OH solution. The mixture stirred overnight at room temperature. Dioxane was removed *in vacuo* and 1.0 M HCl was added to give pH ~ 1. The product was extracted into ethyl acetate. The ethyl acetate solution was washed with 0.2 N HCl, dried over sodium sulfate, filtered, and evaporated to yield a yellow oil (7.7 g, 10.0 mmol, 100%). ESI/MS calculated for $C_{37}H_{63}N_5O_{12} + Na$: 792.4. Found: 792.4.

- Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-boroAlg-pinandediol (SEQ. ID. NO.:11) was prepared by coupling the protected pentapeptide to H-boroAlg-pinandediol. Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-OH (1.8 g, 2.3 mmol) was dissolved 10 mL THF and was cooled to -20°C. Isobutyl chloroformate (0.30 mL, 2.3 mmol) and NMM (0.25 mL, 2.3 mmol) were added. After 5 minutes, this mixture was added to H-boroAlg-pinandediol (0.67 g, 2.3 mmol) dissolved in THF (8 mL) at -20°C. Cold THF (~5 mL) was used to aid in the transfer. Triethylamine (0.32 mL, 2.3 mmol) was added and the reaction mixture was allowed to come to room temperature and to stir overnight. The mixture was filtered and solvent was removed by evaporation. The residue was dissolved in ethyl acetate, washed with 0.2 N HCl, 5% NaHCO₃, and saturated NaCl. The organic phase was dried

5 with Na₂SO₄, filtered, and evaporated to yield a yellow oil.
Half of the crude product (1.5 g) was purified in 250 mg
lots by HPLC using a 4 cm x 30 cm Rainin C-18 reverse phase
column. A gradient from 60: 40 acetonitrile: water to 100%
10 acetonitrile was run over a period of 28 minutes at a flow
rate of 40 mL/min. The fractions containing the desired
product were pooled and lyophilized to yield a white solid
(46 mg). ¹H-NMR (CD₃OD) δ 0.9-1.0 (m, 15H), 1.28 (s, 3H),
1.3 (s, 3H), 1.44 (3s, 27H), 1.6-2.8 (20H), 3.7(m, 1H),
3.9(m, 1H), 4.1-4.7 (7H), 5.05(m, 2H), 5.9(m, 1H). High
res (ESI/MS) calculated for C₅₁H₈₆N₆O₁₃B₁ +H: 1001.635.
Found 1001.633.

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Answer

Preparation of H-Asp-Glu-Val-Val-Pro-boroAlg-pinanediol
ester•trifluoroacetate: The hexapeptide analog, Boc-
20 Asp(O^tBu)-Glu(O^tBu)-Val-Val-Pro-boroAlg-pinanediol, (22.5
mg, 0.023 mmol) was treated with 2 mL of TFA: CH₂Cl₂ (1: 1)
for 2 h. The material was concentrated *in vacuo* and
purified by HPLC using C-18 Vydac reverse phase (2.2 x 25
cm) column with a gradient starting at 60:40
25 acetonitrile/water with 0.1%TFA going to 95:5 over 25
minutes with a flow rate of 8 mL/min. The product eluted
at 80% acetonitrile. The fractions were evaporated and
dried under high vacuum to give 8.9 mg (49%) of the desired
product as white amorphous solid. ¹H-NMR (CD₃OD) δ 5.82
30 (m, 1H), 5.02 (m, 2H), 4.58(m, 1H), 4.42 (m, 3H), 4.18 (m,
4H), 3.90 (m, 1H), 3.62 (m, 1H), 3.01 (dd, 1H), 2.78 (m,
1H), 2.62 (m, 1H), 2.41-1.78 (m, 17H), 1.31 (s, 3H), 1.28
(s, 3H), 1.10 - 0.82 (m, 15H). ESI/MS calculated for
C₃₈H₆₂N₆O₁₁B +H: 789.2. Found: 789.2.